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Analysis of the Oxidative Stress Response of *Penicillium chrysogenum* to Menadione

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The intracellular superoxide and glutathione disulphide concentrations increased in *Penicillium chrysogeum* treated with 50, 250 or 500 μM menadione (MQ). A significant increase in the intracellular peroxide concentration was also observed when mycelia were exposed to 250 or 500 μM MQ. The specific activity of Cu,Zn and Mn superoxide dismutases, glutathione reductase and glutathione S-transferase as well as the glutathione producing activity increased in the presence of MQ while glutathione peroxidase and γ-glutamyltranspeptidase were only induced by high intracellular peroxide levels. The glucose-6-phosphate dehydrogenase and catalase activities did not respond to the oxidative stress caused by MQ.

Keywords: Oxidative stress, menadione, superoxide dismutase, glutathione, glucose-6-phosphate dehydrogenase, *Penicillium chrysogenum*

Abbreviations: DCF, 2′,7′-dichlorofluorescein; Et, ethidium; GPA, glutathione producing activity; GP_x, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulphide; GST, glutathione S-transferase; γGT, γ-glutamyltranspeptidase; G6PD, glucose-6-phosphate dehydrogenase; MQ, menadione; ROS, reactive oxygen species; SOD, superoxide dismutase; *tert*-BOOH, *tert*-butyl hydroperoxide

INTRODUCTION

Fungi frequently encounter high concentrations of harmful reactive oxygen species (ROS) in nature. For example, both brown-rot[1] and white-rot[2] fungi themselves generate considerable amounts of ROS to degrade the components of lignocellulose in wood while fungal pathogens often face ROS formed in the oxidative burst during the phagocyte activation in humans[3] and the infection of plant tissues.[4] In numerous *Aspergillus* and *Penicillium* spp. the flavoprotein glucose oxidase gives rise to significant quantities of peroxide, especially when the microorganisms are grown at high concentrations of glucose.[5] In addition, the industrial penicillin producing fungus *Penicillium chrysogenum* requires high dissolved oxygen concentrations to maintain a satisfactory cellular growth and to fuel the β-lactam biosynthetic machinery with O_2 co-substrate,[6] which may also result in an elevated intracellular formation of ROS in the idiophase when the glutathione content of the cells is low.[7]

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Thus far, the oxidative stress responses which protect organisms form the deleterious effects of ROS have been analysed profoundly in yeasts among fungi, including *Saccharomyces cerevisiae*,[8] *Schizosaccharomyces pombe*,[9] *Candida albicans*[10] and *Hansenula mrakii*.[11] In contrast, surprisingly few data are available on the antioxidative defence systems of filamentous fungi. In an earlier work,[12] we have demonstrated the remarkable resistance of *P. chrysogenum* to the oxidative stress caused by high concentrations of peroxides, which could be explained well with high levels of catalase and glutathione peroxidase activities. In addition, here we present further data on the stress responses of *P. chrysogenum* including the exposure to the superoxide generating agent menadione.

MATERIALS AND METHODS

Culture Conditions, Exposure to Stressors and Monitoring Cell Survival

P. chrysogenum NCAIM 00237 was grown in shake flasks (500 ml) containing 100 ml of a standard culture medium as described elsewhere by Emri *et al.*[7,12–14] The mycelia were separated by filtration on sintered glass, were washed and transferred immediately into a medium (100 ml) containing 101 mM glucose, 1% sodium glutamate, 0.4% K_2HPO_4, 0.2% KH_2PO_4, 0.05% $MgSO_4$ and also supplemented with 50, 250, 500 μM menadione (MQ), 0.5 mM diamide or 0.5 mM *tert*-butyl hydroperoxide (*tert*-BOOH) as required. The starting mycelial dry weight was 5.0 mg ml^{-1} in each experiment, and all the cultures were incubated with shaking at 25°C and at 200 rpm.

Mycelia were harvested at 5 h incubation times by filtration, and cell-free extracts for enzyme activity measurements and protein content determinations were prepared using French-press and centrifugation as described elsewhere.[12] In the γ-glutamyltranspeptidase assay the centrifugation was omitted, and suspensions of disrupted mycelia were used in activity measurements. This enzyme has been shown to appear mainly as a membrane-bound enzyme associated with the central vacuoles in *S. cerevisiae*.[15,16]

For monitoring changes in the intracellular glutathione and ROS concentrations, harvested mycelia were resuspended in ice-cold 5% (w/v) 5-sulfosalicylic acid by vigorous mixing and were left at 4°C for 20 min. After centrifugation at 20000 *g* for 10 min, the supernatants were neutralised with 0.5 M NaOH at 0°C and were immediately analysed.

Cell survival after 5 h exposure to MQ was estimated by washing and transferring mycelia into an oxidant-free culture medium (100 ml) containing 151 mM glucose, 1% sodium glutamate and mineral salts as listed above. Both the rate of glucose utilisation and the changes in the mycelial dry weight were registered.[12,17,18]

Enzyme Activity Determinations, Analytical Procedures and Statistics

Specific glutathione peroxidase (GP_x,[19]), glutathione reductase (GR,[20]), glutathione S-transferase (GST,[21]), γ-glutamyltranspeptidase (γGT,[7]), catalase[22] and glucose-6-phosphate dehydrogenase (G6PD,[13]) activities were measured by the methods indicated in parentheses.

The specific glutathione producing activity (GPA), which is an indicator of the *de novo* glutathione synthesis, was assessed by the method of Murata *et al.*[23] *P. chrysogenum* cells harvested at 5 h incubation time were thoroughly washed and transferred into a culture medium (pH 7.4) consisting of 0.5 M glucose, 20 mM sodium glutamate, 20 mM cysteine, 20 mM glycine, 10 mM $MgCl_2$ and 0.1 M KH_2PO_4. After 1 h incubation with shaking at 25°C and at 200 rpm, cell-free extracts were prepared by 5-sulfosalicylic acid treatment and increases in the intracellular glutathione levels, i.e. the glutathione production values, were determined.

The specific Cu,Zn and Mn superoxide dismutase (Cu,ZnSOD and MnSOD) activities were determined according to Oberley and Spitz.[24] Briefly, in total SOD measurements the reaction mixtures contained 0.2 mM xanthine, 10 U l^{-1} xanthine oxidase (Sigma product number X-4875), 1000 U l^{-1} catalase (Sigma product number C-40), 70 μM Nitro Blue Tetrazolium and 1.4 mM diethylenetriaminepentaacetic acid dissolved in 0.05 M sodium phosphate (pH 7.8) buffer. In the MnSOD assay the reaction mixtures were also supplemented with 4.0 mM NaCN. In all cases the oxidation of Nitro Blue Tetrazolium in the presence and in the absence of cell-free extract was monitored spectrophotometrically at $\lambda = 560$ nm. One unit of SOD activity was defined as the amount of enzyme which inhibited the Nitro Blue Tetrazolium oxidation rate of the control by 50%.

The intracellular glutathione (GSH) and oxidised glutathione (glutathione disulphide; GSSG) concentrations were determined according to Anderson.[25]

To estimate the intracellular peroxide and superoxide levels, 10 μM 2′,7′-dichlorofluorescin diacetate or, in separate experiments, 10 μM dihydroethidium were added to the *P. chrysogenum* cultures at 5 h incubation times. After incubating further for 1 h, the mycelia were harvested, treated with 5-sulfosalicylic acid, and the production of 2′,7′-dichlorofluorescein (DCF; an indicator of peroxide) and ethidium (Et; an indicator of superoxide) were determined spectrofluorimetrically according to Royall and Ischiropoulos[26] and Carter *et al.*,[27] respectively. The extracellular decomposition of both 2′,7′-dichlorofluorescin diacetate and dihydroethidium was tested by incubation of the chemicals in mycelium-free culture fluids which were prepared by filtration at 5 h incubation time. The possible absorption of DCF and Et by hyphae was also thoroughly investigated by addition of DCF and ethidium bromide to the cultures. We concluded that none of these processes interfered significantly with the intracellular peroxide and superoxide determinations.

The protein content of the cell-free extracts was measured by a modification of the Lowry method.[28]

All the experimental data presented here with the exception of cell survival investigations are means of four independent measurements. The variations between experiments were estimated by standard deviations (SD) for each procedure.

Chemicals

2′,7′-Dichlorofluorescin diacetate and dihydroethidium were bought from Molecular Probes Europe, Leiden, The Netherlands. All the other chemicals were purchased from the Sigma-Aldrich Ltd., Budapest, Hungary.

RESULTS AND DISCUSSION

P. chrysogenum mycelia treated with 50 or 250 μM MQ grew and utilised glucose similar to control untreated cultures (Figure 1). Although the fungus also survived the oxidative stress caused by 500 μM MQ the decreased mycelial growth and glucose utilisation rate indicated severe cell injuries in this case (Figure 1). It is notable that while *P. chrysogenum* was found to be exceptionally resistant to oxidative stress caused by high concentrations of either H_2O_2 (0.35–0.70 M) or *tert*-BOOH (0.5–2.0 mM)[12] the MQ tolerance of the fungus was comparable to that observed in rat alveolar epithelial cells[29] and in some yeasts.[9,30]

As shown in Table I, all the MQ concentrations tested increased markedly the intracellular superoxide concentration. Although superoxide anions were disproportionated effectively by SODs the peroxide content of the cells increased significantly only at high, 250 and 500 μM, MQ concentrations (Tables I and II). Most likely, the remarkably high catalase and GP_x activities of the *P. chrysogenum* cells (Table II)[12] prevented the intracellular accumulation of peroxides at 50 μM MQ concentration which did not disturb

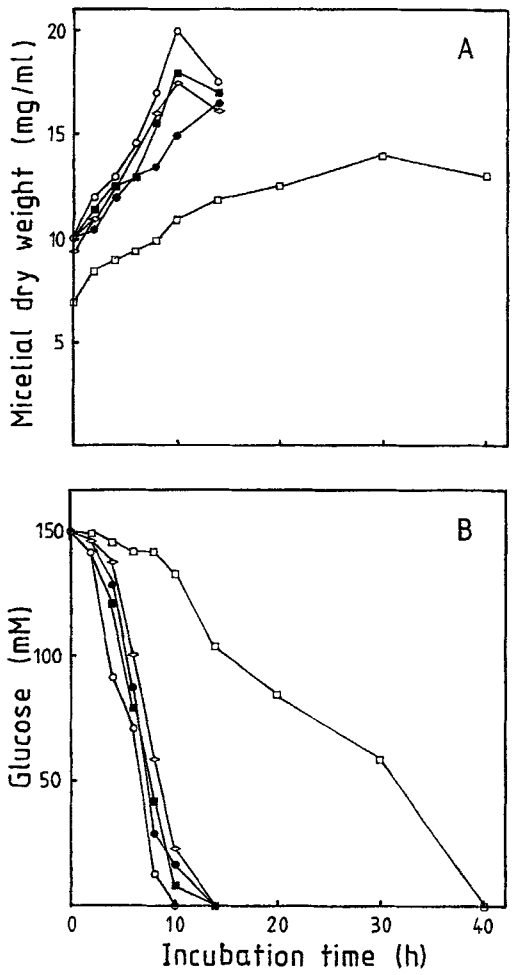

FIGURE 1 Growth (A) and glucose utilisation (B) of MQ- and *tert*-BOOH-treated *P. chrysogenum* mycelia following transfer into oxidant-free culture medium. Equal quantities of mycelia (mycelial dry weights 5.0 mg ml^{-1}) were exposed to 50 μM (■), 250 μM (◇), 500 μM (□) MQ and 0.5 mM *tert*-BOOH + 50 μM MQ (●) for 5 h. Changes in a control culture (○) are also shown. Changes observed in the presence of 0.5 mM *tert*-BOOH, 0.5 mM diamide and 0.5 mM diamide + 50 μM MQ were almost the same as with 50 μM MQ and, therefore, they are not shown for clarity. A typical set of curves is presented here.

the GSH/GSSG redox status of the cells either (Table I).

Similar to other fungi,[31,32] *P. chrysogenum* possessed both Cu,ZnSOD and MnSOD activities, which were induced by MQ in a dose-dependent manner (Table II). In yeasts, the cytoplasmically and possibly peroxisomally located Cu,ZnSOD was shown to be the principal superoxide defence enzyme in the presence of redox cyclers, e.g. MQ and plumbagin[10,33] while the mitochondrial MnSOD of *S. cerevisiae* was regarded as effective against superoxide anions generated by the respiratory chain components of intact mitochondria under normoxic conditions.[34] Although in *P. chrysogenum* the subcellular localisation and the physiological function of SODs have remained yet to be elucidated the contribution of MnSOD to the total SOD activity of the cells, which was 28% in control cultures (Table II), seems to be more significant than in exponentially growing yeasts, where MnSOD accounted for only 5–20% of the total SOD activity under no stress condition.[9,10,33,34]

Interestingly, *P. chrysogenum* SOD activities did not respond to either the addition of 0.5 mM *tert*-BOOH, which increased the intracellular peroxide concentration without affecting the superoxide level and the GSH/GSSG status of the cells, or to the GSH/GSSG redox imbalance generated by 0.5 mM diamide (Tables I and II). In accordance with this observation, *S. pombe* SODs were not induced by 0.2 mM H_2O_2[9] while the addition of 0.4 mM H_2O_2 to growing *C. albicans* cultures increased both the specific Cu,ZnSOD and MnSOD activities.[10]

GSH functions as an effective free radical scavenger inside the cells[35–37] and, furthermore, also plays a crucial role in the detoxification of MQ, which includes two-electron reduction,[38] GSH S-conjugation and transportation of the GSH S-conjugates both outside the cells and into the vacuoles.[39] As a consequence, the specific GST activity as well as the *de novo* synthesis (GPA) and the regeneration (GR) of GSH were induced by all the MQ concentrations tested in *P. chrysogenum* cultures (Table II). Increased GPA and GR activity were also observed in the presence of 0.5 mM *tert*-BOOH and 0.5 mM diamide, i.e. these enzyme activities also responded to oxidative stress caused by peroxides and to the onset of GSH/GSSG redox imbalances (Table II).[12]

TABLE I Changes in the specific GSH, GSSG, DCF and Et concentrations under different culture conditions[a]

Additives	GSH mmol (kg protein)$^{-1}$	GSSG mmol (kg protein)$^{-1}$	Total glutathione[b] mmol (kg protein)$^{-1}$	GSH/GSSG	DCF mmol (kg protein)$^{-1}$	Et mmol (kg protein)$^{-1}$
Control	25 ± 2	2.1 ± 0.3	29 ± 2	12 ± 2	0.019 ± 0.003	0.20 ± 0.03
50 μM MQ	28 ± 3	3.0 ± 0.5*	34 ± 3*	9 ± 2	0.021 ± 0.003	0.27 ± 0.04*
250 μM MQ	28 ± 4	6 ± 1***	40 ± 4***	5 ± 1***	0.025 ± 0.004*	0.9 ± 0.1***
500 μM MQ	19 ± 2**	24 ± 4***	67 ± 4***	0.8 ± 0.2***	0.045 ± 0.006***	1.4 ± 0.2***
0.5 mM *tert*-BOOH	27 ± 2	3.0 ± 0.8*	33 ± 3*	9 ± 2	0.041 ± 0.007***	0.25 ± 0.04
0.5 mM *tert*-BOOH + 50 μM MQ	26 ± 3	11 ± 3***	48 ± 7***	2.5 ± 0.7***	0.040 ± 0.004***	0.35 ± 0.03**
0.5 mM diamide	30 ± 3*	9 ± 2***	48 ± 5***	3 ± 1***	0.020 ± 0.005	0.21 ± 0.03
0.5 mM diamide + 50 μM MQ	28 ± 3	11 ± 3***	50 ± 7***	2.5 ± 0.7***	0.020 ± 0.003	0.29 ± 0.04**

[a]Specific production values are expressed as mean ± SD, calculated from four independent experiments.
[b]Total glutathione concentrations were calculated as GSH + GSSG, where GSSG was expressed in GSH equivalents.
* $p < 5\%$; ** $p < 1\%$; *** $p < 0.1\%$. p values were calculated using the Student's t-test.

Similar to rat alveolar epithelial[29] and cultured Chinese hamster[38] cells, the increased GPA compensated well for the GSH used for antioxidant defence and detoxification in *P. chrysogenum*. As shown in Table I, the total glutathione concentration (GSH + GSSG) increased with increasing MQ concentrations, and the GSH/GSSG redox imbalance observed at 250 μM MQ concentration was exclusively due to the significant increase in the GSSG level. Even in the presence of 500 μM MQ only a moderate 24% decrease in the GSH concentration was detected (Table I). Interestingly, in *S. cerevisiae* the expression of the *GSH1* gene, which encodes 7γ-glutamylcysteine synthetase, has also been shown to be highly inducible by MQ[40] but, in spite of this, the glutathione pool was almost completely depleted by 0.5 mM MQ in less than 0.5 h.[41]

In contrast to the yeasts *S. pombe*[9] and *C. albicans*,[10] neither catalase nor G6PD, the key enzyme of the major NADPH supplier pentose phosphate pathway, were induced by treatment of *P. chrysogenum* mycelia with MQ, and these enzymes were not influenced by peroxides and diamide either (Table II).[12] Furthermore, the specific GP_x and γGT activities were only increased when the intracellular peroxide levels were high, and no enzyme induction was observed in the presence of either 50 μM MQ or 0.5 mM diamide when solely the intracellular superoxide or GSSG concentrations were affected (Tables I and II).

The data presented here and in another paper of ours[12] clearly indicate the considerable antioxidant defence potential of *P. chrysogenum* to cope with oxidative stress caused by either peroxides or superoxide generating agents. According to our preliminary experiments, ROS may also accumulate inside *P. chrysogenum* mycelia under β-lactam producing conditions when high concentrations of the penicillin side-chain precursor phenoxyacetic and phenylacetic acids are added to the fermentation media.[7,42,43] The influx of these aromatic acids into the cells induces GSH-dependent detoxification which results in a substantial decrease in the intracellular GSH levels.[7] Because GSH is thought to be an effective inhibitor of the β-lactam biosynthesis[44,45] this process seems to be advantageous for the antibiotic production. Concomitantly, a range of thioredoxin-dependent enzymes, including broad-specificity disulphide reductase[46] and peroxidase,[47] are likely to compensate for the GSH/GSSG redox imbalance[7] and, therefore, these enzymes may contribute profoundly to the maintenance of a suitable reduced milieu for the penicillin biosynthetic machinery in the idiophase. The physiological investigation of the

TABLE II Changes in the specific GP_x, GR, G6PD, catalase, γGT, GST and SOD activities and GPA values under different culture conditions. Specific activities[a] are expressed in mkat(kg protein)$^{-1}$.

Additives	GP_x	GPA	GR	G6PD	Catalase[b]	γGT	GST	Cu,ZnSOD[c]	MnSOD[c]
Control	0.42 ± 0.06	0.060 ± 0.006	3.1 ± 0.3	2.4 ± 0.2	5.8 ± 0.7	0.14 ± 0.01	0.40 ± 0.05	0.046 ± 0.005	0.018 ± 0.002
50 μM MQ	0.51 ± 0.07	0.075 ± 0.008*	4.4 ± 0.4***	2.5 ± 0.2	5.5 ± 0.8	0.15 ± 0.01	0.56 ± 0.05***	0.056 ± 0.006*	0.024 ± 0.004*
250 μM MQ	0.63 ± 0.07***	0.082 ± 0.009**	4.3 ± 0.4***	2.4 ± 0.3	5.4 ± 0.7	0.14 ± 0.01	0.52 ± 0.04**	0.059 ± 0.007*	0.029 ± 0.004***
500 μM MQ	0.82 ± 0.09***	0.081 ± 0.008**	4.5 ± 0.5***	2.3 ± 0.2	5.6 ± 0.6	0.17 ± 0.02*	0.53 ± 0.04**	0.067 ± 0.007***	0.035 ± 0.005***
0.5 mM *tert*-BOOH	0.73 ± 0.08***	0.080 ± 0.010**	3.8 ± 0.3**	2.4 ± 0.2	5.4 ± 0.8	0.20 ± 0.03**	0.48 ± 0.06	0.048 ± 0.005	0.020 ± 0.002
0.5 mM *tert*-BOOH + 50 μM MQ	0.77 ± 0.07***	0.082 ± 0.009**	4.3 ± 0.4***	2.5 ± 0.2	5.8 ± 0.7	0.22 ± 0.03**	0.56 ± 0.04***	0.060 ± 0.007*	0.028 ± 0.004**
0.5 mM diamide	0.51 ± 0.06	0.087 ± 0.009***	4.7 ± 0.5***	2.5 ± 0.3	5.6 ± 0.6	0.12 ± 0.01	0.47 ± 0.06	0.047 ± 0.005	0.018 ± 0.002
0.5 mM diamide + 50 μM MQ	0.48 ± 0.07	0.085 ± 0.009***	4.6 ± 0.4***	2.3 ± 0.2	5.4 ± 0.7	0.15 ± 0.01	0.57 ± 0.05***	0.055 ± 0.006*	0.025 ± 0.004*

[a]Specific activity values are expressed as mean ± SD, calculated from four independent experiments.
[b]Specific catalase activities are given in kat(kg protein)$^{-1}$.
[c]Specific SOD activities are given in unit(kg protein)$^{-1}$.
*$p < 5\%$; **$p < 1\%$; ***$p < 0.1\%$. p values were calculated using the Student's t-test.

thioredoxin-dependent ROS-eliminating pathways in *P. chrysogenum* is now in progress in our laboratory.

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